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14. ABSTRACT

The goal of this project is to ascertain whether administration of monosodium luminol-GVT (MSL-GVT, an antioxidant drug from Bach Pharma) in a rat model of Gulf war illness (GWI) would alleviate mood and memory dysfunction associated with Gulf war illness (GWI). Specific Aim 1 studies are focused on quantifying the efficacy of different doses of MSL-GVT for suppressing oxidative stress and inflammation and improving neurogenesis in the hippocampus of rats exposed to GWI-related (GWIR) chemicals and stress (GWI-rats). Specific Aim 2 studies are focused on examining whether long-term administration of an apt dose of MSL-GVT would alleviate mood and memory dysfunction and anxiety in GWI-rats, using a battery of behavioral tests. During the past two years, the experiments pertaining to Specific Aim 1 have been completed. This comprises: (1) Exposure of rats to GWIR-chemicals and moderate stress. (2) Oral administration of different doses of MSL-GVT, 4-months after the exposure. (3) Memory and mood function analyses using a battery of behavioral tests. (4) Analyses of oxidative stress and inflammation using biochemical, immunohistochemical and molecular biological studies. The results suggest that administration of higher doses of MSL-GVT treatment to GWI-rats for 8 weeks is efficacious for improving memory and mood function with increased hippocampal neurogenesis and suppression of oxidative stress and inflammation.

15. SUBJECT TERMS
DEET, Gulf war illness, hippocampal neurogenesis, Memory dysfunction, Mood dysfunction, neuroinflammation, oxidative stress, Permethrin, Pyridostigmine bromide, monosodium luminol

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1. INTRODUCTION

Gulf war illness (GWI) is a chronic multi-symptom health problem, which afflicts nearly 30% of veterans who served in the Persian Gulf War-1 (PGW-1). Brain dysfunction, typified by memory dysfunction, depression and anxiety, is one of the major health issues in GWI. While the precise etiology of GWI is unknown, several suspected causes have been proposed. Among these, the hypothesis that GWI is linked to a combination of exposures encountered by service personnel during the war has received much attention. First, veterans who were stationed in the battlefield areas believed to have consumed pills of pyridostigmine bromide (PB) during the war. PB was employed as a prophylactic treatment to protect against a possible attack with organophosphate nerve gas agents. Second, preparations for the PGW-1 comprised measures to offset infectious diseases transmitted by insects/ticks in the region. The measures included the use of pesticides for the area protection and insect repellants on the skin and uniforms. The pesticides included the insecticide permethrin (PM) and the insect repellant DEET. Thus, in view of the exposure of service personnel to the above GWI-related (GWIR) chemicals and war related stress, it is hypothesized that the neurological symptoms displayed by a significant number of PGW-1 veterans are due to synergistic interaction of PB with pesticides PM and DEET and/or stress. This chemical exposure hypothesis is also supported by Research Advisory committee's (RAC's) report on GWI that the overall prevalence of GWI is greater in veterans who used higher amounts of pesticides than veterans who had limited exposure to pesticides during the PGW-1. Consistent with this theory, studies in our laboratory using rat models have shown that combined exposure to low doses of chemicals PB, PM and DEET with mild/moderate stress for 4 weeks causes dysfunction of the hippocampus, which was typified by impairments in memory and mood function with increased oxidative stress, chronic low-level inflammation and greatly declined neurogenesis. Because all of these changes can adversely affect memory and mood function, drugs capable of suppressing oxidative stress and inflammation and/or increasing neurogenesis have received attention for alleviating cognitive and mood impairments in veterans afflicted with GWI. This project is focused on ascertaining the efficacy of an antioxidant and anti-inflammatory drug monosodium luminol-GVT (MSL-GVT from Bach Pharma) for easing memory and mood dysfunction in a rat model of GWI.

2. KEYWORDS

Anxiety
DEET
Gulf war illness
Hippocampal
neurogenesis
Memory dysfunction
Mood dysfunction
Neuroinflammation
Oxidative stress
Permethrin
Pyridostigmine
bromide
Monosodium luminol GVT

3. ACCOMPLISHMENTS

3.1. Major Goals:

The major goal of this project is to examine the efficacy of monosodium luminol-GVT (MSL-GVT from Bach Pharma) for alleviating mood and memory dysfunction in a rat model of

GWI. The chosen animal model of GWI has been well characterized, simulates the various exposures likely encountered by the veterans during the PGW-1, and reliably induces cognitive and mood dysfunction in association with increased oxidative stress, low-level chronic inflammation and declined neurogenesis in the hippocampus. Studies in Specific Aim 1 are focused on quantifying the efficacy of oral administration of different doses of MSL-GVT (via oral gavage) for suppressing oxidative stress and inflammation, and stimulating the proliferation of hippocampal neural stem cells (NSCs) and increasing the extent of net neurogenesis in rats exposed to GWI-related (GWIR)-chemicals and moderate levels of stress four months earlier (GWI-rats). The goal is to identify an optimal dose of MSL-GVT that greatly suppresses inflammation and oxidative stress and enhances hippocampal neurogenesis in rats exposed to GWIR-chemicals and moderate levels of stress. Studies in Specific Aim 2 are focused on determining whether oral administration of an apt dose of MSL-GVT for prolonged periods (12 weeks) is efficient for alleviating mood and memory dysfunction in rats exposed to GWIR-chemicals and stress six months earlier, using a battery of behavioral tests.

3.2. Studies Accomplished During the Past Two Years:

3.2.1. Specific activities pertaining to Aim 1 studies:

The experiments in Aim 1 comprises 5 major groups of rats:
  Group 1: GWI-rats receiving MSL-GVT at 40mg/Kg b.w.
  Group 2: GWI-rats receiving MSL-GVT at 80mg/Kg b.w.
  Group 3: GWI-rats receiving MSL-GVT at 160mg/Kg b.w.
  Group 4: GWI-rats receiving vehicle (VEH)
  Group 5: Age-matched naïve control rats

3.2.1.1. Animal numbers, survival and tissue harvesting:

Seventy-three (73) rats have been used so far in 2 different cohorts for Aim 1 studies.

(a) The first cohort comprised 31 animals at the start of experiments. From these, 29 animals reached the endpoint of experiments. Two animals were found dead during the four- months waiting period between the exposure of animals (to Gulf war illness related (GWIR) chemicals and 15 minutes of restraint stress for 28 days) and the commencement of MSL-GVT treatment. The brain tissues from 29 animals have been harvested and biochemical and molecular biological studies have been performed. These animals belong to the following groups:

(1) GWI-MSL 40mg/Kg, n=6   (2) GWI-MSL 80mg/Kg, n=6
(3) GWI-MSL 160mg/Kg, n=5   (4) GWI-vehicle (VEH), n=6
(5) Naive control, n=6

(b) The second cohort comprised 42 animals at the start of experiments. From these, 39 animals reached the endpoint of experiments. Two animals were found dead during the four- months waiting period between the exposure of animals to GWIR chemicals and stress and the commencement of MSL-GVT treatment. An additional animal was euthanized in this period because it developed uncontrolled seizures, typified by continuous Stage-V seizures (bilateral forelimb clonus with rearing and falling). The brain tissues from 39 animals have been harvested and various immunohistochemical studies have been performed. These animals belong to the following groups:

(1) GWI-MSL 40mg/Kg, n=8   (2) GWI-MSL 80mg/Kg, n=8
(3) GWI-MSL 160mg/Kg, n=8   (4) GWI-VEH, n=8
(5) Naive control, n=7

3.2.1.2. Time-line of various procedures for animals belonging to Aim 1 studies:

(i) Exposure period to GWIR-chemicals and stress: 28 days (daily)
(ii) Survival period between exposure and treatment: 4 months
(iii) MSL-GVT or VEH treatment period: 8 weeks (5 times/week)
(iv) 5'-bromodeoxyuridine (BrdU) injection period: 5 days (in the 3rd week of treatment)
(iv) Cognitive and mood function tests   Started from 5th week of treatment
(v) Euthanasia and Tissue harvesting: After 8 weeks of treatment

3.2.1.3. Brief description of procedures performed so far:

(a) Exposure of animals to GWIR-chemicals and stress: Animals were exposed daily to the following chemicals for 28 days: Pyridostigmine bromide (PB) at 2 mg/kg/day (via oral gavage), DEET at 60 mg/kg/day (via dermal application) and Permethrin at 0.2 mg/kg/day (via dermal application). In addition, animals were subjected daily to 15 minutes of restraint stress using rat restrainers during the above 28-day period.

(b) Survival period between exposure and treatment: Following the exposure to GWIR- chemicals and stress, animals were maintained in the vivarium for four months in regular cages (two per cage) with ad libitum access to food and water.

(c) Administration of MSL-GVT or VEH: Treatment was given for 8 weeks (5 times/week) via oral gavage, commencing in the 5th month after exposure to GWIR-chemicals and stress. The doses of MSL-GVT employed were 40 mg/Kg, 80 mg/Kg and 160 mg/Kg.

(d) BrdU injections: Subgroups of rats from all groups received BrdU injections in the 3rd week of drug/vehicle treatment daily for 5 days at a dose of 100 mg/Kg/day.

(e) Behavioral tests for assessing cognitive and mood function: We examined animals in all groups through multiple stress-free behavioral tests. These include pattern separation test (PST), novel object recognition test (NORT) and object location test (OLT) for assessing cognitive and memory function, and sucrose preference test (SPT) and modified novelty suppressed feeding test (NSFT) for assessing the extent of depressive-like behavior.

(f) Euthanasia and tissue harvesting: Animals belonging to cohort 1 were deeply anesthetized with isoflurane in a small chamber, until the animal ceased respiration. Deeply anesthetized animals were decapitated following thoracotomy and brain tissues were dissected rapidly for biochemical and molecular biological studies. Animals belonging to cohort 2 were first deeply anesthetized with isoflurane and then perfused through the heart with 4% paraformaldehyde solution. Fixed tissues were harvested for histological studies.

(g) Analyses of oxidative stress:
The hippocampal and/or cerebral cortical tissues obtained from animals belonging to cohort #1 were used for the following measurements:
(i) Analyses of the expression of oxidative stress response and antioxidant genes using "The Rat Oxidative Stress Response PCR Array" from Qiagen: We analyzed the expression of 84 key genes involved in oxidative stress response and antioxidant activity in the hippocampus of animals belonging to different groups using quantitative real time PCR (qRT-PCR), to ascertain the effects of MSL-GVT treatment on oxidative stress.
(ii) Quantification of 3-nitrotyrosine: Increased modification of tyrosine residues in proteins to 3-nitrotyrosine by peroxynitrite or other potential nitrating agents is seen in tissues subjected to oxidative stress. Hence, we quantified 3-nitrotyrosine in hippocampal tissue

extracts from different groups, using the nitrotyrosine ELISA Kit.

(iii) Measurement of lipid peroxidation through quantification of malondialdehyde (MDA) and 4-hyrdoxynoneal (4-HNE): Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as MDA, a natural bi-product of lipid peroxidation. 4-HNE is another bi-product of lipid peroxidation. We measured MDA and 4-HNE in hippocampal and/or cortical tissue extracts from different groups using TBARS Assay Kit for MDA or ELISA Kits for MDA and 4-HNE.

(iv) Measurement of SOD-2 (MnSOD, a marker of oxidative stress) and NRF-2 concentrations in the hippocampus: MnSOD and NRF-2 are reliable markers of oxidative stress. We measured these markers using ELISA Kits.

(h) Analyses of inflammation in the hippocampus via measurement of inflammatory cytokines: The hippocampal tissues obtained from animals belonging to cohort #1 were also used for measurement of the relative levels of inflammatory cytokines in different groups of animals. We employed "The Rat Cytokine Plate Array" from Signosis, which facilitated analyses of 16 rat cytokines in a high-throughput manner. The cytokines included: tumor necrosis factor-alpha ((TNF-alpha), interleukin-1 alpha (IL-1alpha), interleukin-1 beta (IL-1beta), vascular endothelial growth factor (VEGF), fibroblast growth factor beta (FGFb), interferon gamma (IFN gamma), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-15 (IL15), leptin, monocyte chemoattractant protein-1 (MCP-1), IFN-gamma-inducible protein 10 (IP-10 or CXCL10), stem cell factor (SCF), Regulated on Activation, Normal T Cell Expressed and Secreted (Rantes), Macrophage inflammatory protein 1 alpha (MIP-1a) and transforming growth factor-beta (TGFbeta).

(i) Analyses of inflammation in the hippocampus via quantification of reactive astrocytes and activated microglia: Fixed brain tissues obtained from animals belonging to cohort #2 were processed for cryostat sectioning. Serial sections (every 20th) through the entire hippocampus were processed for immunohistochemical detection of glial fibrillary acidic protein, (GFAP, a marker of astrocytes) and ED-1 (a marker of activated microglia). We quantified the area fraction of GFAP+ immunoreactive elements in different subfields (dentate gyrus, and CA1 and CA3 subfields) of the hippocampus using J Image. We also measured the total number of ED-1+ elements (activated microglia) in the entire hippocampus using stereology (optical fractionator method).

(j) Measurement of net hippocampal neurogenesis in the third week of VEH or MSL-GVT treatment: A set of serial sections (every 15th) through the entire hippocampus was processed for detection of BrdU+ cells (i.e. newly born cells) using immunohistochemistry. Another set of serial sections was processed for measurement of the neuronal differentiation of BrdU+ newly born cells, using dual immunofluorescence for BrdU and neuron-specific nuclear antigen (NeuN) and Z-sectioning in a Nikon confocal microscope. We measured the total number of BrdU+ cells in the subgranular zone-granule cell layer (SGZ-GCL, neurogenic area) of the hippocampus using stereology (optical fractionator method) and the percentage of BrdU+ cells expressing NeuN using serial Z-sections in a confocal microscope. Using the total number of BrdU+ cells per SGZ-GCL and the percentage of BrdU+ cells expressing NeuN, we calculated the net hippocampal neurogenesis for the period of BrdU injection (i.e. in the 3rd week of treatment).

(k) Measurement of the status of hippocampal neurogenesis and neural stem cell activity at the end of 8 weeks of VEH or MSL-GVT treatment: A set of serial sections (every 15th) through the entire hippocampus was processed for the detection of doublecortin (DCX). DCX is a marker of newly born neurons. As the expression of DCX in newly born neurons lasts for ~two weeks in rats, DCX immunostaining visualizes neurons that are born in the two weeks prior to euthanasia. Hence, it provides a good measure of ongoing status of hippocampal neurogenesis. We quantified the total number of DCX+ newly born neurons in the SGZ-GCL of the hippocampus using stereology (optical fractionator method).

This quantification provided information on the status of hippocampal neurogenesis at the end of 8 weeks of VEH or MSL-GVT treatment. We also quantified neural stem cell activity through Ki-67 immunostaining and Sox-2 and Ki-67 dual immunofluorescence and confocal microscopy.

3.2.2. Specific activities pertaining to Aim 2 studies:

The experiments in Aim 2 comprises 4 major groups of rats:

Group 1: GWI-rats receiving no treatment
Group 2: GWI-rats receiving VEH treatment
Group 3: GWI-rats receiving MSL-GVT at 160mg/Kg b.w. for 12 weeks
(NOTE: Highest dose of MSL-GVT was chosen based on the results of Aim 1 studies)
Group 4: Age-matched naïve control rats

3.2.2.1. Animal numbers, survival and tissue harvesting:

A cohort of rats (n=46) has been employed so far for Aim 2 studies. From these, 34 animals were exposed to GWIR-chemicals and 15 minutes of restraint stress for 28 days. Twelve rats were maintained as naive control rats. One animal was found dead during the six-months waiting period between the exposure of animals (to GWIR-chemicals and stress) and the commencement of treatment. Thus, 45 animals reached the endpoint of experiments. The brain tissues from these animals have now been harvested for immunohistochemical studies. These animals belong to the following four groups:

(1) GWI rats receiving no treatment, n=11
(2) GWI rats receiving VEH treatment, n=11
(3) GWI rats receiving MSL 160 mg/Kg, n=11
(4) Naive control rats, n=12

3.2.2.2. Time-line of various procedures for animals belonging to Aim 2 studies:

(i) Exposure period to GWIR-chemicals and stress: 28 days (daily)
(ii) Survival period between exposure and treatment: 6 months
(iii) MSL-GVT or VEH treatment period: 12 weeks (5 times/week)
(iv) 5'-bromodeoxyuridine (BrdU) injection period: 5 days (in the 3rd week of treatment)
(iv) Cognitive and mood function tests   Started from 8th week of treatment
(v) Euthanasia and Tissue harvesting: After 12 weeks of treatment

3.2.2.3. Brief description of procedures performed so far:

(a) Exposure of animals to GWIR-chemicals and stress: This was done as described above for Aim 1 studies (see section 3.2.1.3.)

(b) Survival period between exposure and treatment: Following the exposure to GWIR- chemicals and stress, animals were maintained in the vivarium for six months in regular cages (two per cage) with ad libitum access to food and water.

(c) Administration of MSL-GVT or VEH: Treatment was given for 12 weeks (5 times/week) via oral gavage, commencing in the 7th month after exposure to GWIR-chemicals and stress. The dose of MSL-GVT employed was 160 mg/Kg. This dose was chosen based on its highest efficacy in Aim 1 studies.

(d) BrdU injections: Subgroups of rats from all groups received BrdU injections in the 8th week of drug/vehicle treatment daily for 5 days at a dose of 100 mg/Kg/day.

(e) Behavioral tests for assessing cognitive and mood function: Subgroups of animals belonging to different groups were subjected to multiple behavioral tests. These include pattern separation test (PST), novel object recognition test (NORT), object location test (OLT) and water maze test (WMT) for assessing cognitive and memory function, and sucrose preference test (SPT), modified novelty suppressed feeding test (NSFT) for assessing the extent of depressive-like behavior. Additional animals will be raised for different groups and examined with these tests in the coming year. Once data from all animals belonging to different groups are available, data will be computed and statistically analyzed in the coming year.

(f) Euthanasia and tissue harvesting: Animals that were raised so far (n=45) were deeply anesthetized with isoflurane and perfused through the heart with 4% paraformaldehyde solution. Fixed tissues were harvested for histological studies.

In the coming year, these tissues will be cut into 30-micrometer thick sections and serial sections will be used various histological studies. This will include analyses of inflammation in the hippocampus via quantification of reactive astrocytes and activated microglia, measurement of net hippocampal neurogenesis (for the 8th week of treatment) and measurement of the status of hippocampal neurogenesis at the end of 12 weeks of VEH or MSL-GVT treatment.
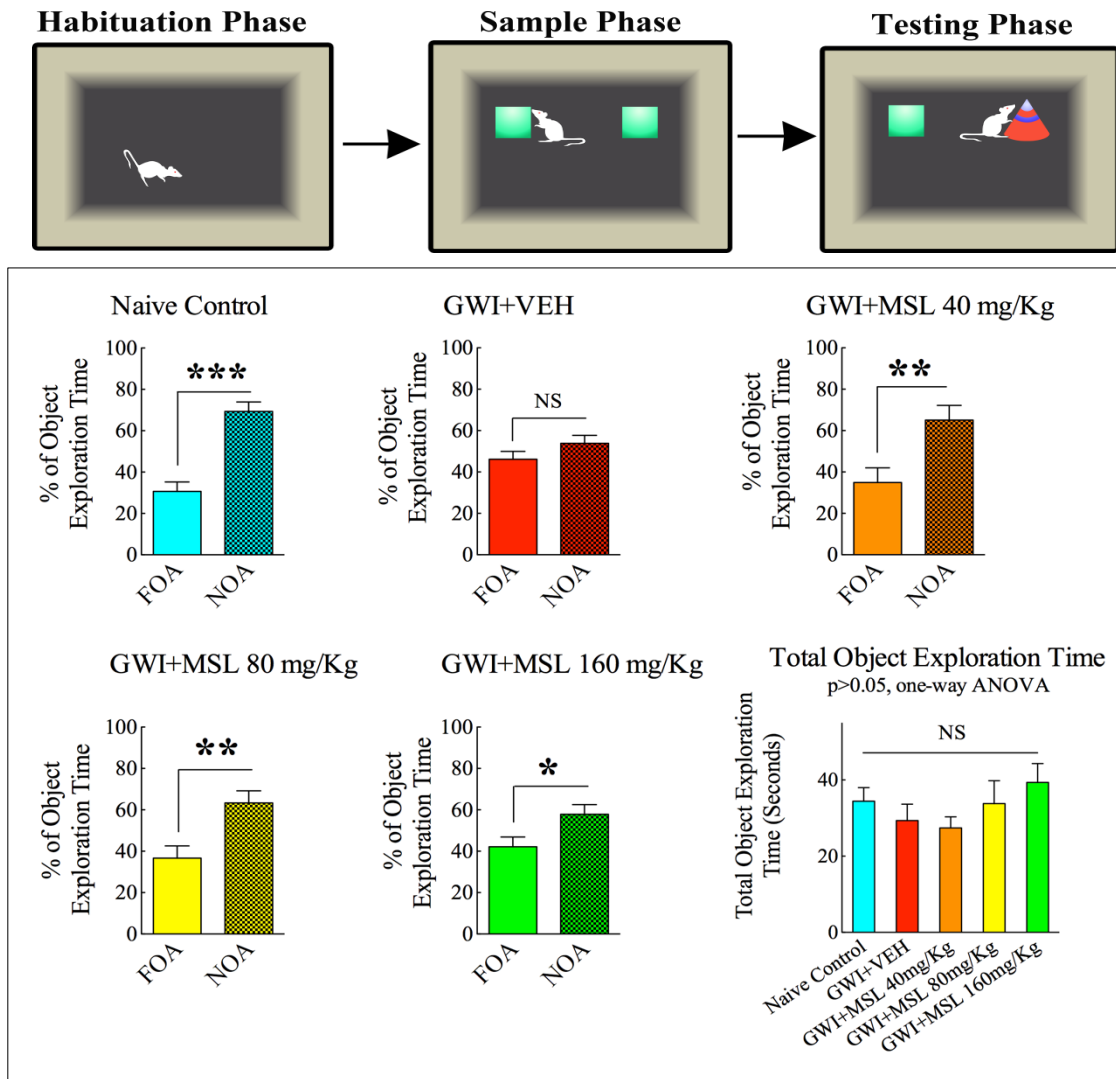
3.2.3. Progress details:

Dose-response studies using MSL-GVT in GWI rats (Aim 1 studies) suggest the following findings:

3.2.3.1. MSL-GVT treatment improved both cognitive and memory function in GWI rats:

(a) MSL-GVT treatment at a higher dose improved pattern separation function in GWI rats: In the previous year's report, we provided the results of pattern separation test (PST). Pattern separation function reflects proficiency for discriminating analogous experiences through storage of similar representations in a non-overlapping manner (Leutgeb et al., Science, 315: 961-966, 2007; Yassa and Stark, Trends Neurosci, 34: 515-525). Maintenance of this function depends upon the integrity of the hippocampus particularly normal levels of neurogenesis. In this test, each rat successively explored two different sets of identical objects (object types 1 and 2) placed on distinct types of floor patterns (Patterns 1 and 2 [P1 and P2]) for 5 minutes each in the two acquisition trials (separated by 30 minutes). Thirty minutes later, in the testing phase (Trial-3), each rat explored an object from trial 2 (which is now a familiar object) and an object from Trial-1 (which is now a novel object) placed on the floor pattern employed in trial 2 (P2). We demonstrated that GWI rats receiving VEH or low doses of MSL-GVT (40/80 mg/Kg) displayed impaired pattern separation function but a higher dose MSL-GVT (160 mg/Kg) reversed pattern separation dysfunction in GWI rats. *Please refer to the previous year's report for details on this finding.* We now present the results of two additional cognitive and memory tests in sections "b" and "c" below.

(b) MSL-GVT treatment reversed recognition memory dysfunction in GWI rats: Recognition memory function depends mainly on the integrity of the perirhinal cortex and partly on the hippocampus. This function was measured using a novel object recognition test (NORT), as described in our published report (Hattiangady et al., Frontiers in Behavioral Neuroscience, 2014). We examined rats belonging to different groups with 30 min delay between the object exploration phase (which involved exploration of two identical objects for 5 minutes in an arena, i.e. "Sample Phase") and the "Testing Phase" (which involved exploration of objects in the same arena as in the exploration phase but with replacement of one of the objects with a new object). The "Testing phase" was video-recorded and analyzed using Noldus-Ethovision software. GWI rats receiving VEH showed inability for novel object

discrimination as they spent similar percentages of time exploring the familiar and novel objects (Fig. 1). In contrast, GWI rats receiving different doses of MSL-GVT (40, 80 or 160 mg/Kg) spent greater percentages of their object exploration time with the novel object, akin to naive control rats (Fig. 1). *Thus, MSL-GVT treatment, even at lower doses, reverses recognition memory dysfunction.*
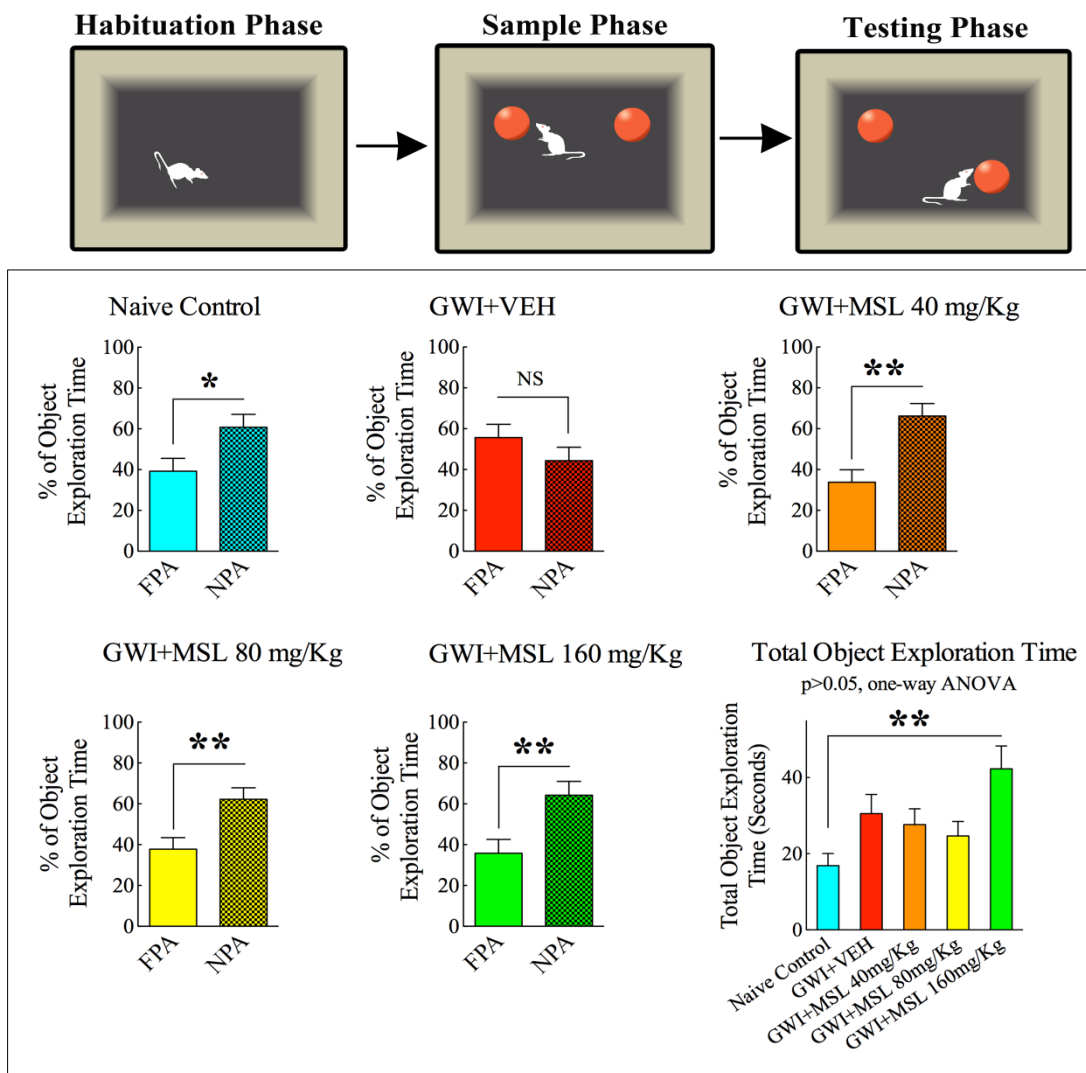

Figure 1 - Results of Novel Object Recognition Test (NORT). Top panel illustrates the sequence of trials in this test. The first five bar charts compare percentages of exploration time spent in the familiar object area (FOA) and the novel object area (NOA) in different animal groups. Only animals that explored objects >8 seconds in the testing phase were included (n=10-14/group). Cyan, Naive control group; red, GWI+VEH group; orange, GWI+MSL 40 mg/Kg group; yellow, GWI+MSL 80 mg/Kg group; green, GWI+MSL 160 mg/Kg group. The bar chart on lower right corner compares the total object exploration time between groups in the testing phase. One-way ANOVA analysis did not show differences between groups, implying that the specificity of the novel object exploration time (time spent in NOA) was not influenced by differences in the total object exploration time. *, P<0.05; **, p<0.01; ***, p<0.001; NS, not significant.

(c) MSL-GVT treatment improved object location memory function in GWI rats: *Cognitive ability to detect subtle changes in the environment* (such as a minor change in the location of an object) was measured via an object location test (OLT). Maintenance of this function depends upon the integrity of the hippocampus circuitry. Each animal had 3 successive trials. The first trial (habituation phase) involved placement of the animal in the center of the empty open field box; animal was allowed to explore for 5 minutes and then placed in its home cage. After an inter-trial interval of 30 min, animal was placed again in the open field with two similar objects placed on right and left sides (sample phase). Animal was

allowed to freely explore the objects for 5 min and then placed in its home cage. Thirty minutes later, animal was placed again in the same box with the left side object placed in its original position and the right side object moved to another corner (Testing phase or Trial 3). Animal was allowed to explore for 5 min, and the entire Trial 3 was video-recorded and analyzed using Noldus-Ethovision software. *The choice to explore the object displaced to a novel location reflects the ability of animal to discern minor changes in the location of objects in its immediate environment.* GWI rats receiving VEH displayed impairment in this cognitive function, as they did not show affinity for the object moved to a novel place in Trial 3. Rather, they spent nearly equal amounts of time with the object in the familiar place (FP object) and the object in the novel place (NP object). In contrast, GWI rats receiving different doses of MSL-GVT (40, 80 or 160 mg/Kg) spent greater percentages of their object exploration time with the object in the novel place, similar to naive control rats (Fig. 2). *Thus, MSL-GVT treatment, even at lower doses, reverses location memory dysfunction.*
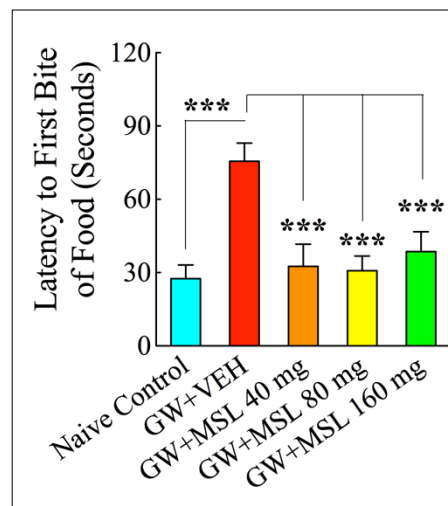

Figure 2 - Results of Object location Test (OLT). Top panel illustrates the sequence of trials in this test. The first five bar charts compare percentages of exploration time spent with the object in the familiar area (or familiar place area, FPA) and the object in the novel area (or novel place area, NOA) in different animal groups. Only animals that explored objects >8 seconds in the testing phase were included (n=9-13/group). Cyan, Naive control group; red, GWI+VEH group; orange, GWI+MSL 40 mg/Kg group; yellow, GWI+MSL 80 mg/Kg group; green, GWI+MSL 160 mg/Kg group. The bar chart on lower right corner compares the total object exploration time between groups in the testing phase. One-way ANOVA analysis showed that GWI rats receiving MSL at 160 mg/Kg explored objects for greater amount of time than other groups, which may imply a greater anxiolytic effect of higher dose of MSL in GWI rats. *, P<0.05; **, p<0.01; NS, not significant.

3.2.3.2. MSL-GVT treatment improved mood function in GWI rats:

(a) MSL-GVT treatment at moderate to higher doses reversed anhedonia in GWI rats: In the previous year's report, we provided the results of sucrose preference test (SPT), which is a test for measuring anhedonia (i.e. inability to feel pleasure in activities that normally bring pleasure, a measure of depression). We demonstrated that GWI rats receiving VEH or lower doses of MSL-GVT (40 mg/Kg) displayed anhedonia. However, GWI rats treated with moderate to higher doses of MSL-GVT (80-160 mg/Kg) reversed anhedonia. *Please refer to the previous year's report for details on this finding.* We now present the result of an additional depression related test in section "b" below.

(b) MSL-GVT treatment reversed mood dysfunction in GWI rats: We assessed motivational deficit or mood dysfunction in GWI rats using a modified novelty suppressed feeding test (NSFT). This test provides a reliable measure of motivation level or depressive-like behavior in animals that resemble those in humans. Rats were first subjected to fasting for 23 hrs (by withdrawing food pellets from the cage) but were allowed to drink water. A single trial test was next conducted in an empty rat cage. Food pellets were placed in a corner of the cage on a white paper. Each rat was next released at the opposite corner of the cage and allowed to explore the cage for five minutes. In the absence of depression, rats subjected to fasting move quickly towards the food pellets and start eating. In contrast, rats with depression either take much longer time to reach and eat the food or lack motivation to move towards the food. Thus, latency to the first bite of food served as a measure of the extent of motivation in this test. GWI rats receiving VEH exhibited greater latencies to eat food than naïve control rats (Fig. 3), which implied that they have mood dysfunction or motivational deficit. In contrast, GWI rats receiving different doses of MSL-GVT (40, 80 or 160 mg/Kg) moved quickly towards food pellets and started eating. Latencies to the first bite of food in these rats were comparable to that seen in naive control rats (Fig. 3).

Figure 3 - Results of a modified Novelty Suppressed Feeding Test (NSFT). The latency to the first bite of food is greatly increased in GWI rats receiving VEH, in comparison to naive control rats. Interestingly, in GWI rats receiving different doses of MSL-GVT, latency values to the first bit of food are comparable to that seen in naive control rats and greatly reduced in comparison to GWI rats receiving VEH, implying normalization of mood function in GWI rats with MSL-GVT treatment. Statistically significant outliers were removed in all groups through a Grubb's test. Data presented are from 13-14 rats in each group.***, $p < 0.001$
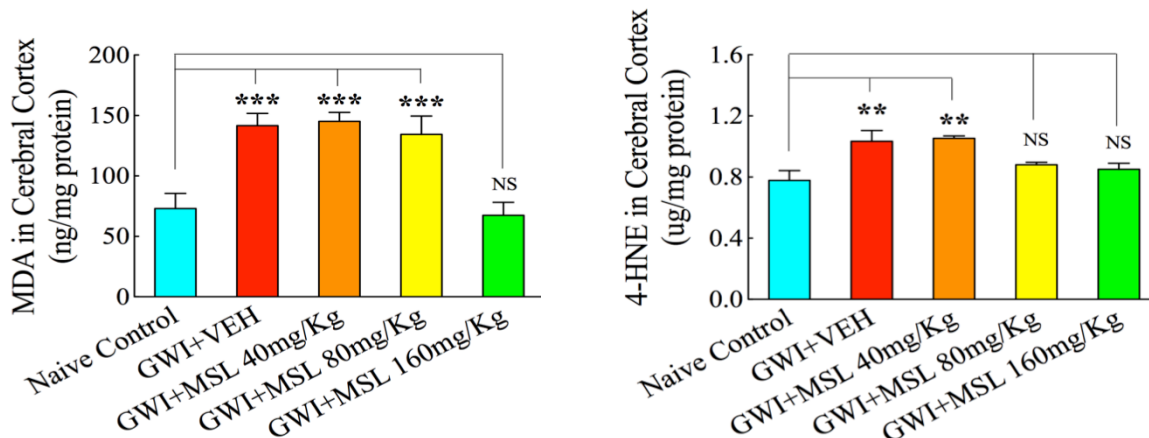
3.2.3.3. MSL-GVT treatment reduced oxidative stress in the hippocampus and cerebral cortex of GWI rats:

(a) *MSL-GVT treatment normalized the expression of genes related to oxidative stress in the hippocampus:* In the previous year's report, we provided the results of the expression of oxidative stress response and antioxidant genes: We analyzed the expression of 84 key genes involved in oxidative stress response and antioxidant activity in the hippocampus of animals belonging to different groups using quantitative real time PCR (qRT- PCR). Among 84 genes related to oxidative stress response examined in this experiment, GWI rats receiving VEH exhibited increased expression of 24 genes, in comparison to age-matched naive control animals, implying the presence of significant oxidative stress in the hippocampus of GWI rats. Among these 24 genes, the expression of 4 genes was completely normalized by MSL-GVT treatment. The genes comprise: peroxiredoxin-6 protein (Prdx6), manganese-dependent superoxide dismutase (MnSOD or Sod2), sequestosome1 (Sqstm1) and sulfiredoxin-1 (Srxn1). Furthermore, 20 genes that displayed increased expression in GWI rats were normalized to control levels by higher doses of MSL-GVT treatment (80 or 160 mg/Kg). These include Cat gene encoding catalase protein, Ctsb gene encoding cathepsin B, Dhcr24 gene encoding 24-dehydrocholesterol

reductase, Gsr gene encoding glutathione reductase, Gstk1 gene encoding glutathione s-transferase kappa 1, Gstp1 encoding glutathione s-transferase-1, Idh1 gene encoding isocitrate dehydrogenase 1, Ncf1 encoding neutrophil cytosolic factor 1 protein, Prdx1-4 genes encoding peroxiredoxins 1-4, Prnp gene encoding prion protein, Ptgs2 gene encoding prostaglandin-endoperoxide synthase (also known as cyclooxygenase, Slc38a1 gene encoding solute carrier family 38, Txn1 gene encoding thioredoxin 1, Txnip encoding thioredoxin interacting protein, Txnrd1 gene encoding thioredoxin reductase 1, Txnrd2 gene encoding thioredoxin reductase 2, and Ucp2 gene encoding uncoupling protein 2. Overall, our qRT-PCR analyses suggested that MSL-GVT treatment alleviates oxidative stress in the hippocampus. *Please refer to the previous year's report for details on this oxidative stress gene expression study.*
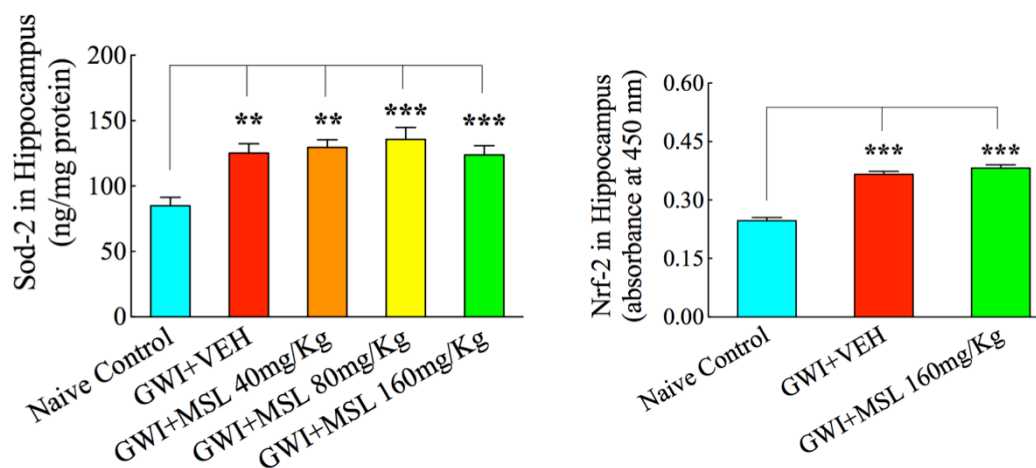
(b) MSL-GVT treatment was associated with reduced concentration of MDA in the hippocampus of GWI rats: In the previous year's report, we provided results on concentrations of MDA and 3-nitrotyrosine (3-NT) in the hippocampus. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as MDA, a natural bi-product of lipid peroxidation. MDA concentration in GWI rats receiving VEH was higher than in naive control rats. However, GWI rats receiving higher dose of MSL-GVT (160 mg/Kg) displayed reduced MDA concentration than GWI rats receiving VEH, implying that administration of a higher dose of MSL-GVT (160 mg/Kg) decreased oxidative stress in the hippocampus. Increased modification of tyrosine residues in proteins to 3-nitrotyrosine by peroxynitrite or other potential nitrating agents is seen in tissues subjected to oxidative stress. 3-NT levels did not differ between GWI rat groups however. *Please refer to the previous year's report for details on MDA and 3-NT levels in the hippocampus.* We now present results of additional experiments on MDA and 4-HNE (another bi-product of lipid peroxidation) in the cortex of GWI rats (see below for details).

(c) MSL-GVT treatment reduced the concentration of MDA and 4-HNE in the cerebral cortex of GWI rats: Higher concentrations of MDA and 4-HNE were observed in the cerebral cortex of GWI rats receiving VEH, in comparison to naive control rats (Fig. 4). However, GWI rats receiving higher doses of MSL-GVT (160 mg/Kg for MDA, 80 or 160 mg/Kg for 4-HNE) displayed similar concentrations of MDA and 4-HNE as naive control rats (Fig. 4), implying that administration of a higher dose of MSL-GVT (160 mg/Kg) can normalize oxidative stress in the cerebral cortex.

Figure 4 - Results of MDA and 4-HNE ELISA assays for the cerebral cortex. MDA concentration is increased in GWI rats receiving VEH and GWI rats receiving 40 or 80 mg MSL. However, in GWI rats receiving 160 mg MSL, MDA concentration is similar to that seen in naive control animals. The concentration of 4-HNE is increased in GWI rats receiving VEH and GWI rats receiving 40 MSL. However, in GWI rats receiving 80 or 160 mg MSL, the concentration of 4-HNE is similar to that seen in naive control animals. Thus, a higher dose of MSL normalizes both MDA and 4-HNE levels in the cortex. Data presented are from 6 rats in each group **, p<0.01; ***, p<0.001.

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(d) MSL-GVT treatment did not normalize increased levels of Nrf-2 and Sod2 in the hippocampus of GWI rats: Since multiple genes relevant to oxidative stress (including Sod2) showed significantly upregulated expression in GWI rats receiving VEH and normalized expression in GWI rats receiving a higher dose of MSL-GVT, we investigated whether such changes were also reflected in assays of other markers of increased oxidative stress. We first measured the concentration of Sod2 (superoxide dismutase-2, also known as manganese dependent superoxide dismutase, MnSod), an antioxidant that typically shows upregulation in conditions such as increased oxidative stress and hence serves as a reliable marker of increased oxidative stress. As expected, GWI rats receiving VEH exhibited increased concentration of Sod2 but MSL-GVT treatment (both low and high doses) did not normalize Sod2 levels in the hippocampus (Fig 5, left panel). We next measured Nrf2 (nuclear factor [erythroid-derived 2]-like 2) in the hippocampus. Nrf2 is a transcription factor that is well recognized for its role in regulating the manifestation of antioxidant proteins that guard against oxidative damage elicited by injury or inflammation. Nrf2 is typically upregulated in conditions such as increased oxidative stress to limit the detrimental effects of reactive oxygen species. This assay also revealed similar results: GWI rats receiving VEH exhibited increased concentration of Sod2 but MSL-GVT treatment (both low and high doses) did not decrease Nrf2 concentration in the hippocampus (Fig 5, right panel). Thus, MSL-GVT maintains redox homeostasis in the GWI rat brain (evidenced through decreased levels of MDA and 4-HNE) without altering the brain's innate defense mechanisms against increased oxidative stress, which is evinced by increased concentrations of Sod-2 and Nrf-2 concentrations in the hippocampus.
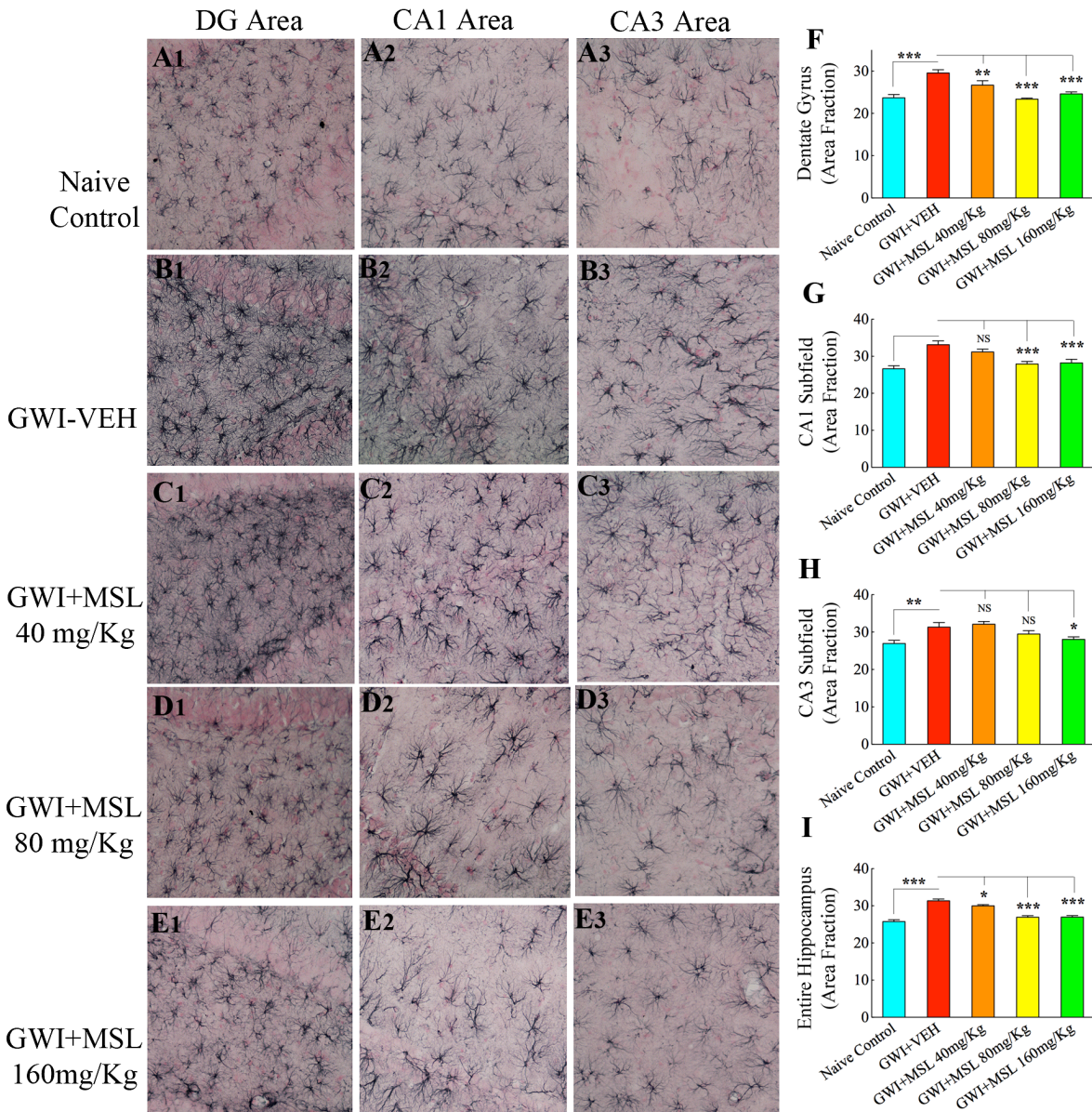

Figure 5 - Results of Sod-2 (left panel) and Nrf-2 (right panel) assays for the hippocampus. The concentrations of both Sod-2 and Nrf-2 were increased in GWI rats receiving VEH. None of the MSL-GVT doses decreased the concentrations of these two proteins however. Data presented are from 5-6 rats in each group. **, $p < 0.01$; ***, $p < 0.001$

3.2.3.4. MSL-GVT treatment reduced inflammation in the hippocampus of GWI rats:

In the previous year's report, we provided results on the relative levels of inflammatory cytokines in the hippocampus of different groups of animals. We employed "The Rat Cytokine Plate Array" from Signosis, which facilitated analyses of 16 rat cytokines in a high-throughput manner. The cytokines included: TNF-alpha, IL-1alpha, IL-1beta, VEGF, FGFb, IFN gamma, IL-5, IL-6, IL15, leptin, MCP-1, IP-10 (or CXCL10), SCF, Rantes, MIP-1a and TGFbeta. This study revealed no significant differences in the concentration of most of these cytokines between naive control animals, GWI rats receiving VEH, and GWI rats receiving different doses of MSL-GVT. *Please refer to the previous year's report for details on this assay.* We now present results of immunohistochemical analyses of inflammation in the hippocampus (see below).

(a) MSL-GVT treatment normalized astrocytic hypertrophy in the hippocampus: We measured the area fraction of GFAP+ astrocytes in different subfields of the hippocampus. This analysis showed increased density of GFAP+ astrocytic elements (soma and processes) in all subfields (dentate gyrus, CA1 and CA3 subfields) of the hippocampus of GWI rats
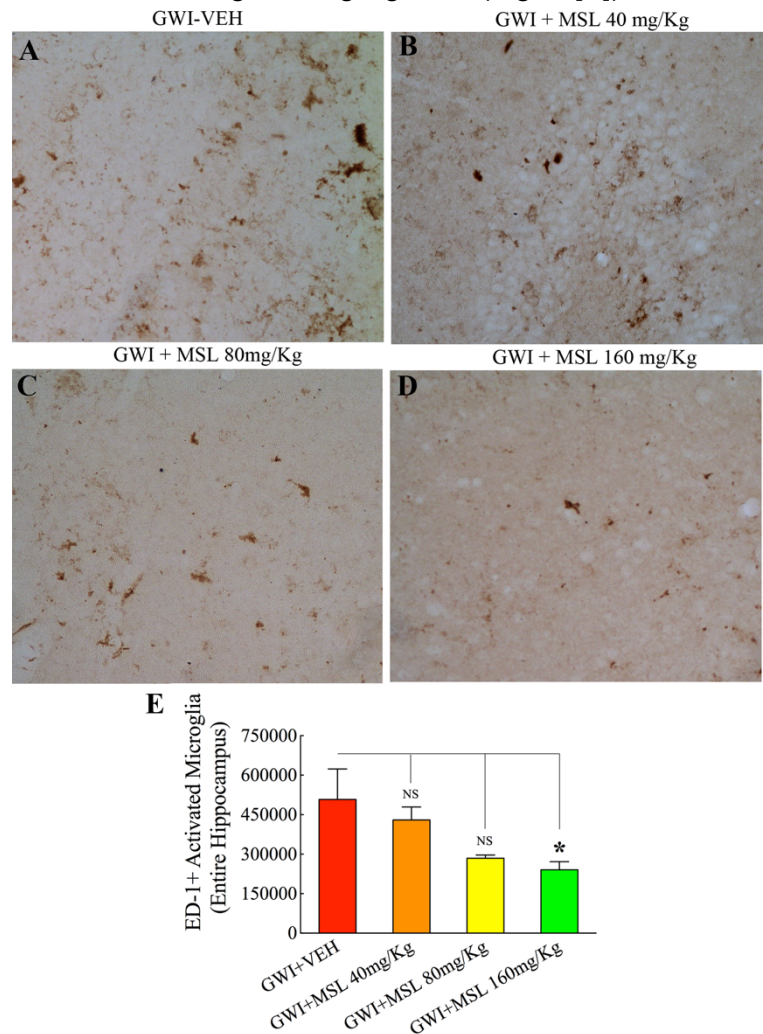
receiving VEH, implying hypertrophy of astrocytes (a sign of mild chronic inflammation). In contrast, GWI rats receiving different doses of MSL-GVT exhibited normalized density of GFAP+ astrocytic elements (Fig. 6). All doses of MSL-GVT normalized astrocytes in the dentate gyrus (Fig. 6 [F]), 80 and 160 mg doses normalized astrocytes in the CA1 subfield (Fig. 6 [G]) and only 160 mg dose normalized astrocytes in the CA3 subfield (Fig. 6 [H]). When the hippocampus is taken in its entirety, all doses of MSL-GVT normalized astrocytes. Thus, MSL-GVT treatment reverses astrocyte hypertrophy in GWI rats.

Figure 6 - Figures A1-E3 illustrate GFAP+ astrocytic elements in the dentate gyrus (A1, B1, C1, D1, E1), CA1 subfield (A2, B2, C2, D2, E2) and CA3 subfield (A3, B3, C3, D3, E3) of the hippocampus from a naive control rat (A1-A3), GWI rats receiving VEH (B1-B3) or MSL-GVT at 40 mg/Kg (C1-C3), 80mg/Kg (D1-D3) or 160mg/Kg (E1-E3). Bar charts in F-I compare the area fraction of GFAP+ elements in the dentate gyrus (F), CA1 subfield (G), CA3 subfield (H) and the entire hippocampus (I). Note reductions in the area fraction of GFAP+ elements in GWI rats receiving MSL in comparison to GWI rats receiving VEH. Data presented are from 7-8 rats in each group *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$
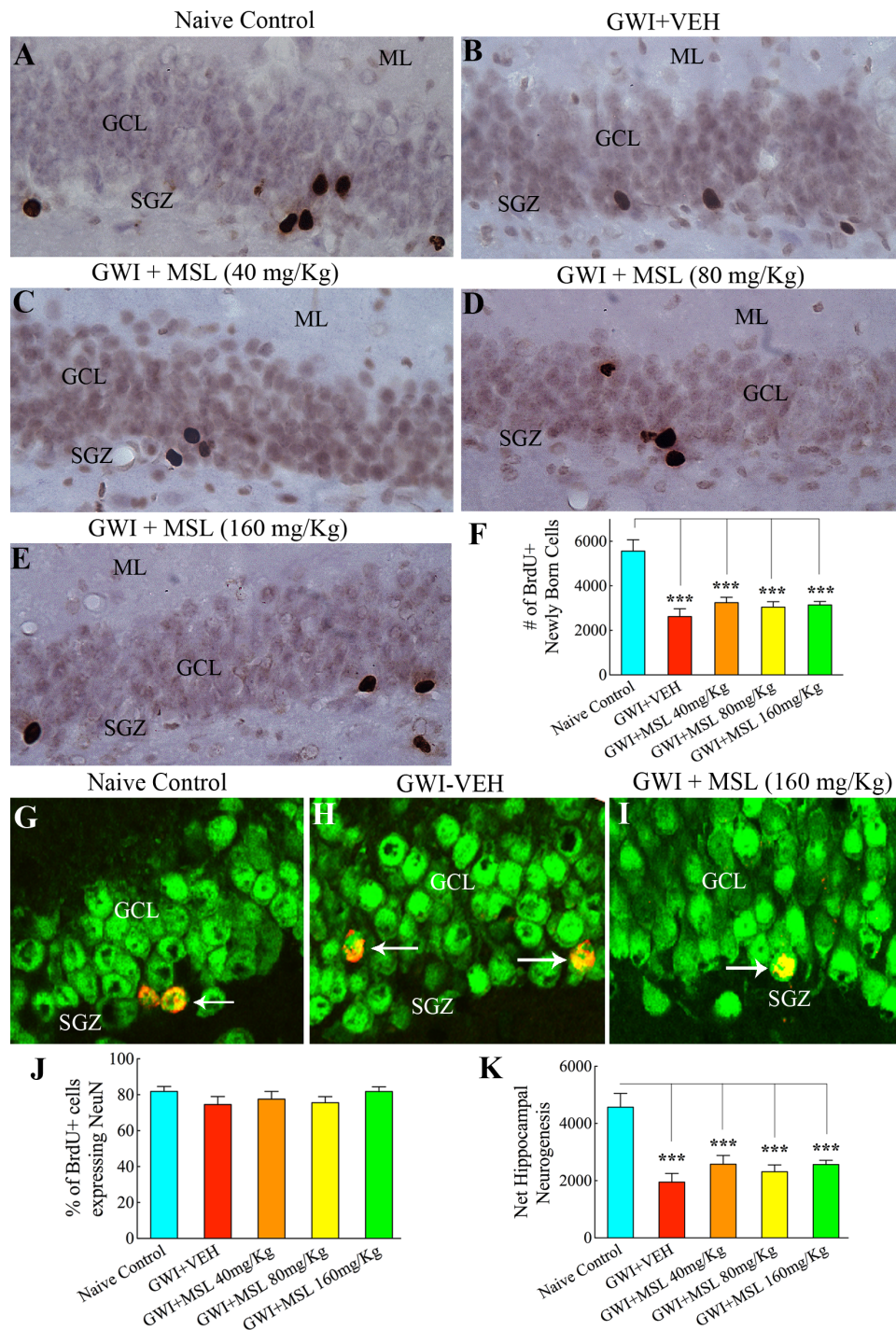
(b) MSL-GVT treatment reduced the numbers of activated microglia/macrophages in the hippocampus: We measured the numbers of ED-1+ structures (a marker of activated microglia and macrophages) in the entire hippocampus using stereology. This quantification revealed the presence of a large number of ED-1+ structures in all subfields of the

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hippocampus of GWI rats receiving VEH. Figure 1 [A] illustrates the presence of such structures in the dentate gyrus of the hippocampus. Treatment of GWI rats with MSL-GVT for 8 weeks decreased the numbers of ED-1+ structures in the hippocampus (Fig. 7). The decrease was statistically significant in GWI rats receiving 160 mg/Kg MSL (Fig. 7 [E]).

Figure 7 - Figures A-D illustrate ED-1+ structures (activated microglia/macrophages) in the hippocampus of GWI rats receiving VEH (A), GWI rats receiving MSL at 40 mg/Kg (B), 80mg/Kg (C) or 160mg/Kg (D). Bar chart in E compares numbers of ED-1+ structures in the entire hippocampus. Note a significant reduction in the number of ED-1+ elements in GWI rats receiving a higher dose of MSL (160 mg/Kg), in comparison to GWI rats receiving VEH. Data presented are from 8 rats in each group. NS, not significant; *, $p<0.05$.
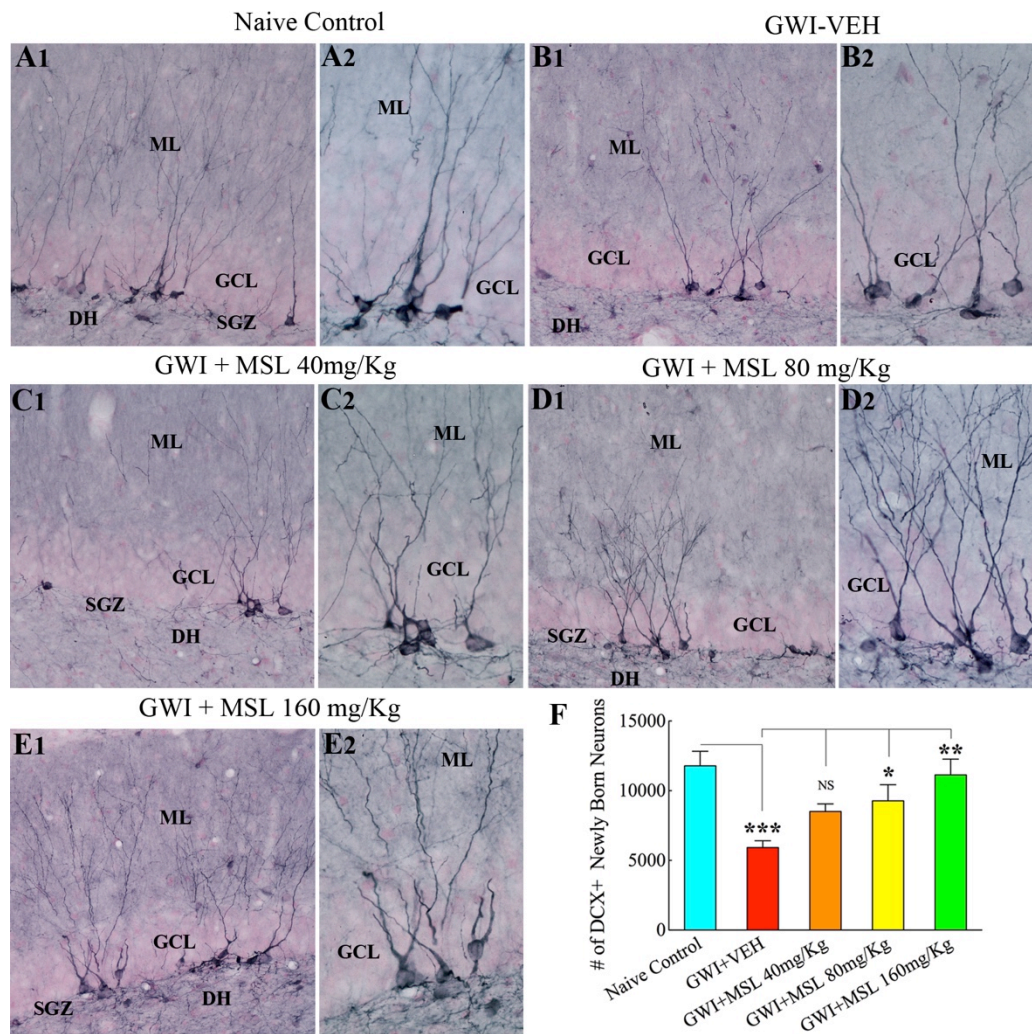
3.2.3.5. Short-term (3 weeks of) MSL-GVT treatment did not increase neurogenesis in the hippocampus of GWI rats: We quantified hippocampal neurogenesis through in vivo labeling with BrdU (a thymidine analog that labels all dividing cells in the S-phase of cell cycle and hence serves as a birth-dating marker). GWI rats received daily injections of BrdU for five days in the 3rd week of VEH or MSL treatment, which labeled cells that were born in the neurogenic region (SGZ-GCL) of the hippocampus in the 3rd week of treatment. Quantification of BrdU+ cells and fractions of BrdU+ cells expressing the mature neuronal marker NeuN in the SGZ-GCL facilitated the quantification of net hippocampal neurogenesis (Fig. 8 [A-F]). Quantification of BrdU labeled cells demonstrated decreased production of newly born cells in the SGZ-GCL of GWI rats receiving VEH, in comparison to naive control rats (Fig. 8 [A-F]). This declined production of newly born cells did not improve with 3 weeks of MSL-GVT treatment, as all MSL-GVT treated groups exhibited BrdU+ cell numbers comparable to GWI rats receiving VEH. Quantification of the neuronal differentiation of newly born cells demonstrated similar extent of conversion of newly born cells into neurons in all groups (Fig. 8 [G-J]). Measurement of net hippocampal neurogenesis (using BrdU+ cell numbers and percentages of neuronal differentiation of BrdU+ cells) revealed decreased neurogenesis in GWI rats receiving VEH as well as GWI rats receiving different doses of MSL-GVT, in comparison to age-matched naive control rats. **Thus, short-term (3 weeks of) MSL-GVT treatment is not efficacious for enhancing neurogenesis in the hippocampus.**

Figure 8 - Figures in A-E illustrate BrdU+ cells in the SGZ-GCL of the hippocampus from a naive control rat (A), and GWI rats receiving VEH (B) and MSL at 40 mg/Kg (C), 80mg/Kg (D) or 160mg/Kg (E). Bar chart in F compares numbers of BrdU+ newly born cells in the SGZ-GCL of the hippocampus between different groups of rats. Note a significant reduction in the number of BrdU+ cells in GWI rats receiving VEH or different doses of MSL, in comparison to naive control rats. Figures in G-I illustrate examples of BrdU+ cells expressing NeuN (arrows) from a naive control rat (G), a GWI rat receiving VEH (H) and a GWI rat receiving 160 mg/Kg MSL. The bar chart in J compares percentages of BrdU+ cells expressing NeuN whereas the bar chart in K compares net hippocampal neurogenesis between different groups. Note a significant reduction in net hippocampal neurogenesis in GWI rats receiving VEH or different doses of MSL, in comparison to naive control rats. Data presented are from 7-8 rats in each group ***, $p < 0.001$. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

3.2.3.6. Long-term (8 weeks of) MSL-GVT treatment normalized neurogenesis in the hippocampus of GWI rats:

We evaluated DCX+ newly born neurons and their numbers in the SGZ-GCL of the hippocampus using stereology (optical fractionator method) (Fig. 9). This quantification provided information on the status of hippocampal neurogenesis at the end of 8 weeks of VEH or MSL-GVT treatment. This quantification revealed decreased production of newly born neurons in the SGZ-GCL of the hippocampus in GWI rats receiving VEH, in comparison to naive control rats (Fig. 9). In contrast, GWI rats receiving higher doses of MSL (80 or 160 mg/Kg) displayed enhanced neurogenesis, in comparison to GWI rats receiving VEH (Fig. 9). In GWI rats receiving 160 mg/Kg MSL, the extent of neurogenesis was comparable to age-matched naive control rats (Fig. 9 [F]). **Thus, long-term (8 weeks of) MSL-GVT treatment at a higher dose normalizes hippocampal neurogenesis in GWI rats.**
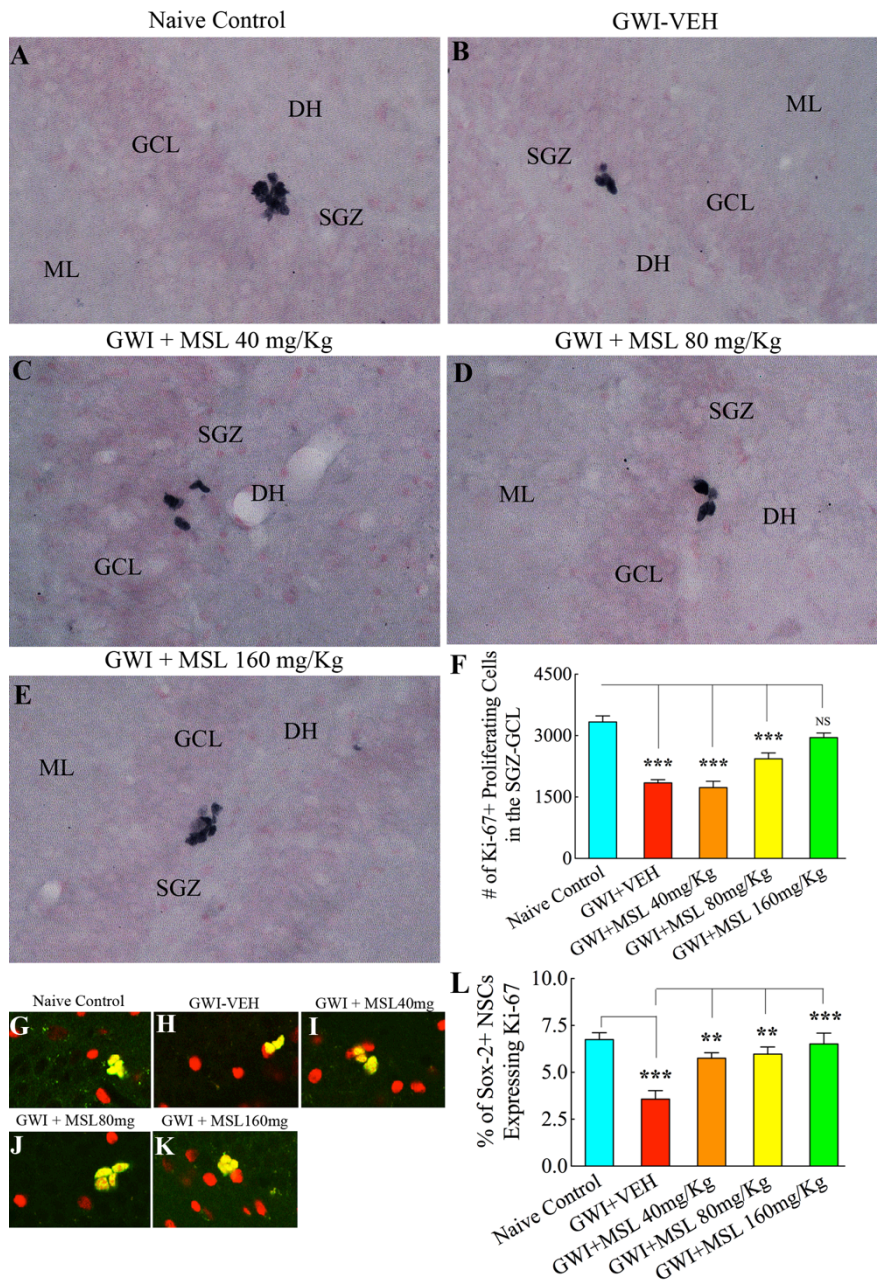

Figure 9 - Figures in A1-E2 illustrate DCX+ newly born neurons in the SGZ-GCL of the hippocampus from a naive control rat (A1), and GWI rats receiving VEH (B1) and MSL at 40 mg/Kg (C1), 80mg/Kg (D1) or 160mg/Kg (E1). A2, B2, C2, D2 and E2 are magnified views of regions from A1, B1, C1, D1 and E1. Bar chart in F compares numbers of DCX+ newly born neurons in the SGZ-GCL of the hippocampus between different groups of rats. A significant reduction in the number of DCX+ neurons is seen in GWI rats receiving VEH, in comparison to naive control rats. However, MSL-GVT treatment for 8 weeks at higher doses (80 or 160 mg/Kg) improved neurogenesis in the hippocampus. Data presented are from 6-7 rats in each group *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

3.2.3.7. Long-term (8 weeks of) MSL-GVT treatment normalized neural stem cell activity in the hippocampus of GWI rats:
We first quantified neural stem cell activity in the SGZ-GCL through immunostaining for Ki67 (a marker of proliferating cells in all phases of cell cycle) and

stereological quantification of Ki67+ cells using serial sections through the entire hippocampus. This revealed that GWI rats receiving VEH displayed reduced proliferation of neural stem cells (Fig. 10). However, MSL-GVT treatment for 8 weeks at a higher dose (160 mg/Kg) normalized the proliferation rate of neural stem cells (Fig. 10). Low dose treatment did not significantly improve neural stem cell activity however. Next, we quantified the fractions of putative neural stem cells in the SGZ (i.e. cells expressing the transcription factor Sox-2) expressing Ki-67. This also showed greatly reduced neural stem cell activity in GWI rats receiving VEH. However, GWI rats receiving MSL-GVT displayed improved activity of neural stem cells. The overall activity in GWI rats receiving a higher dose of MSL-GVT (160 mg/Kg) was comparable to that seen in age-matched naive control rats. **Thus, long-term (8 weeks of) MSL-GVT treatment at a higher dose normalizes hippocampal neural stem cell activity in GWI rats.**

Figure 9 - Figures in A-E illustrate Ki67+ cell clusters (i.e. proliferating neural stem cells) in the SGZ-GCL of the hippocampus from a naive control rat (A), and GWI rats receiving VEH (B) and MSL at 40 mg/Kg (C), 80mg/Kg (D) or 160mg/Kg (E). Bar chart in F compares numbers of Ki-67+ proliferating neural stem cells in the SGZ-GCL of the hippocampus between different groups of rats. A significant reduction in the number of Ki-67+ neural stem cells is seen in GWI rats receiving VEH, in comparison to naive control rats. However, MSL-GVT treatment for 8 weeks at a higher dose (160 mg/Kg) improved this activity. Data presented are from 6 rats in each group. Figures G-K illustrate examples of Sox-2+ putative neural cells expressing Ki-67 in different groups. Bar chart in L compares percentages of Sox-2+ cells expressing Ki-67 (i.e. proliferating neural stem cells) in the SGZ-GCL of the hippocampus between different groups of rats. A significant reduction in the proliferation of neural stem cells is seen in GWI rats receiving VEH, in comparison to naive control rats. However, MSL-GVT treatment for 8 weeks improved neural stem cell activity. Data presented are from 5 rats in each group *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

3.3. Opportunities for Training and Professional Development:

Nothing to Report

3.4. Dissemination of Results to Communities of Interest:

We will write an original research article using data from Aim 1 studies and submit it for publication in a peer-reviewed journal.

3.5. Plans for the Next Reporting Period:

In the coming year, we plan to perform the following experiments:
(1) We will write an original research article using data from Aim 1 studies and submit it for publication in a peer-reviewed journal.
(2) We will raise another cohort of GWI rats, which will involve exposure of animals to GWIR-chemicals and stress for 28 days, long-term (12 weeks of) MSL-GVT treatment (at 160mg/Kg) commencing 6-months after exposure to GWIR-chemicals and stress, analyses of cognitive, memory and mood function through a battery of behavioral tests and harvesting of tissues for various biochemical studies.
(3) Tissues that have already been harvested from Aim 2 studies will be used for various immunohistochemical studies and quantification. These will include: (a) Measurement of ED-1+ activated microglial cells in the hippocampus using stereological cell counting. (b) Quantification of the area fraction of GFAP+ structural elements in different subfields of the hippocampus using J Image. (c) Proliferation of neural stem cells (NSCs) in the subgranular zone SGZ using: (i) Ki-67 and Sox-2 dual immunofluorescence and confocal microscopic analyses. (d) Addition of newly born cells over a period of 5 days (in the 8th week of treatment) via stereological counting of BrdU+ cells in the subgranular zone-granule cell layer (SGZ-GCL) of the dentate gyrus (DG) using serial sections through the hippocampus. (e) Differentiation of new cells added to the SGZ-GCL into neuron-specific nuclear antigen+ (NeuN+) mature neurons using BrdU and NeuN dual immunofluorescence and confocal microscopic analyses. (f) Net hippocampal neurogenesis by utilizing data such as the total numbers of BrdU+ cells in the SGZ-GCL and the percentages of BrdU+ newly born cells that differentiate into mature NeuN+ neurons.

4. IMPACT:

The results obtained from Aim 1 studies suggest that oral administration of MSL-GVT for 8 weeks at a relatively higher dose (160 mg/Kg) is efficacious for alleviating cognitive and memory impairments as well as mood dysfunction in GWI rats. Interestingly, the improvements in cognitive, memory and mood function were associated with suppression of oxidative stress and inflammation and enhancement of hippocampal neurogenesis. However, additional studies as proposed in Aim 2 of this project are needed for making clear conclusions on the efficacy of MSL-GVT for treating chronic GWI. In Aim 1 studies, MSL-GVT treatment commenced 4 months after the exposure to GWIR-chemicals and stress whereas in Aim 2 studies, MSL-GVT treatment will commence at 6 months after the exposure to GWIR-chemicals and stress. Assessment of the effects of any treatment commencing at a delayed time-point after the exposure to GWIR-chemicals and stress has greater significance since >25 years have passed since the exposure occurred to veterans of PGW-1.

5. CHANGES AND PROBLEMS:

(i) Changes in approach:

An amendment was made to the animal protocol during the past year, which was approved by the IACUC and the ACURO. None anticipated for the coming year.

(ii) Actual or Anticipated Problems or Delays and Plans to Resolve them:

Our Institution has moved from the Temple campus of TAMU College of Medicine to the College Station campus of TAMU College of Medicine. This has resulted in resubmission of the animal protocol for approval by the IACUC at College Station. The IACUC at College Station has already approved this protocol. The approved protocol has been sent to the ACURO for approval before commencing experiments in the new location. This would likely slow down the remaining long-term Aim 2 animal experiments to be completed in year 3. Hence, some additional time (beyond the end date of September 29, 2017) will be required to complete studies proposed in this project.

(iii) Changes that had a significant impact on expenditures:

Nothing to Report

(iv) Significant Changes in the use of vertebrate animals or biohazards:

Nothing to Report

(v) Significant Changes in the Care of Vertebrate Animals:

Nothing to Report

6. PRODUCTS:

Publications:

Nothing to Report

7. PARTICIPANTS AND OTHER COLLABORATIVE ORGANIZATIONS

The following research staff members from PI's laboratory were compensated from this grant (for the percentage of effort contributed to this project) during the past year.

Personnel	Role	Percent Effort
Ashok K. Shetty	Principal Investigator	25%
Bharathi Hattiangady	Assistant Research Professor	25%
Bing Shuai	Senior Research Associate	100%

Other Collaborators:

Paul Wong (Retired Professor, MD Anderson Cancer Center, Smithville, TX)
Dr. Wong has performed consultation duties to this project during the past two years (as

described in the original proposal). He has provided advise on handling and preparation of MSL-GVT solution for oral gavage and the dosage of MSL-GVT administration, as he had extensively worked on this drug in other animal models earlier. He is also overseeing the timely supply of MSL-GVT from Bach Pharma for these studies.

Changes in active other support of the PI or Key Personnel:

There have been no changes in the effort level of PI to this grant. However, there will be changes in the effort level of key research personnel for this project during the coming year. Dr. Shuai's effort will be reduced to 50% effective October 1, 2016 because of his involvement in another project. Dr. Hattiangady has left our Institution. To compensate these, two post-docs will contribute a portion of their effort (25-50% effort) to this project in the coming year. One post-doc (Dr. Raghavendra Upadhya) will commence his 25% effort from October 1, 2016 and the other post-doc (Dr. Maheedhar Kodali) will commence his 50% effort from March 1, 2017.

Other Organizations Involved in this Project:

Bach Pharma Company provided MSL-GVT for no cost for these studies through our consultant Dr. Paul Wong. However, Bach Pharma is not involved in the research design, conduct of experiments or analyses and interpretation of data in this project. This study is entirely performed from funds from the Department of Defense grant (W81XWH-14-1-0572). Hence, there is no conflict of interest to disclose.

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None